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Prenatal DEHP exposure induces hippocampal neurotoxicity in male offspring via PTEN dysregulation and impaired Akt/mTOR and NMDA signaling

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Abstract

Widespread human exposure to phthalates is caused by their intensive usage in industrial and consumer plastic products. DEHP (di(2-ethylhexyl) phthalate) is one of the most often used phthalates and is presented not only in food and fluids but also in the air and dust contact with plastic products. Regrettably, phthalates easily migrate into the human body and act as potent toxicants, mainly on endocrine and metabolic status. In the last decade, several epidemiological studies have indicated a correlation between prenatal exposure to phthalates and adverse effects on neurodevelopment in offspring. Our research aimed to assess the impact of DEHP prenatal subchronic exposure on male offspring's behavior and learning ability and identify the primary target brain structure/s of neurotoxic action. Heightened anxiety in male offspring was evident through increased rearing, frequent line crossings, hurried movements, and reduced grooming behavior. These behaviors were accompanied by a decline in recognition memory and diminished interest in exploring novel objects. Obtained data showed that prenatal oral exposure to DEHP in a selected concentration induces irreversible changes in brain structures of the male offspring, primarily in the hippocampus, that underlies significant alterations in cognitive behavior and enhanced anxiety. The molecular mechanism of DEHP-induced hippocampal neurotoxicity in the maturing male brain involves changes in phosphatase and tensin homolog (PTEN) subcellular location, which suppresses Akt/mTOR signaling, enhances GluN2B NMDA mediated synapse depression and decreases mitochondrial fusion.

Keywords: DEHP, Hippocampus, Neurotoxicity, Prenatal exposure, PTEN.

1. Introduction

Phthalates are a group of chemicals that are added to plastic products to enhance their sustainability, plasticity, and durability. These chemicals occur in hundreds of products, including cosmetics, vinyl flooring, food packaging, toys, and paint. Phthalates are 1,2- 1,2-benzene dicarboxylic acid esters produced from phthalic anhydride with the addition of alcohol and a catalyst [1]. Since these small molecules are not chemically bound to main polymers, they easily migrate to the environment. Because of the large production volume, wide application, and great viability of phthalates' sources (water, air, sediments, soil, food, and so on), the presence of phthalates became almost ubiquitous. Human exposure to ortho-phthalates is widespread since they easily migrate out of products. Recent findings have shown significant concentrations of various phthalates in different biological fluids, including amniotic fluid, breast milk, saliva, blood, and urine [2]. Indeed, biomarkers of phthalate exposure are detected in more than 98% of the U.S. population [3]. Simultaneous exposure to several phthalates starts in utero and continues throughout life (childhood, puberty, and adulthood).

Since personal care products taken by a pregnant female are a substantial source of human phthalate exposure, prenatal exposure's effect on developmental plasticity should be studied and assessed. Perinatal exposure to some phthalates is associated with increased vulnerability to anxiety- and depressive-like behaviors. Prenatal and perinatal exposure is of particular attention because of a very low rate of xenobiotics' metabolism at an early stage of development. Children have been reported as having the highest exposures specifically to DEHP, DBP, BBP, and DnOP9 [4, 5]. Certain populations, such as individuals with high dietary exposure or those undergoing medical procedures using DEHP-containing equipment, may experience higher-than-average DEHP intake.

Since certain ortho-phthalates are established endocrine disruptors linked to a host of adverse reproductive and metabolic outcomes across the lifespan, widespread population exposure is an unsettled issue [6, 7]. Phthalate adverse effects are also connected with obesity, decreased female fertility, early labor, and low weight in the neonatal period. Along with all that, there is evidence that phthalates influence allergy and asthma symptoms [5]. Aside from these disorders, findings suggest the neurotoxic effects of phthalates. The association of phthalate exposure with al-

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tered neurobehaviors in animals, including impaired self-righting ability, has been reported earlier [8, 9]. Evidence shows that prenatal exposure to these chemicals adversely affects brain development, leading to cognitive and motor impairments in early childhood, as well as behavioral issues such as poor executive function, attention deficits, working memory challenges, delayed language development, reduced IQ, and an increased risk of ADHD in both preschool and later childhood. Research conducted across 11 different countries has examined the potential effects of prenatal exposure to phthalates on children's development Scientists monitored children for possible neurodevelopmental changes using environmental assessments and validated biomarkers. The findings indicated that children whose mothers had the highest levels of DEHP metabolites in their urine during pregnancy were nearly three times more likely to be diagnosed with ADHD compared to those whose mothers had the lowest levels [10]. Additionally, epidemiological study from around the world indicates that prenatal and early postnatal exposure to phthalates is strongly linked to several neurodevelopmental issues. These include impaired language development, changes in neurocognitive behavior, disturbances in infant visual recognition memory, and symptoms of neurodevelopmental disabilities such as autism spectrum disorder and ADHD [11–14].

However, data on their neurotoxic effects and the underlying molecular mechanisms remain limited, and recent research highlights the need for further analysis [15, 16]. The high lipophilicity of phthalates and their potential to penetrate the BBB, together with behavior disorders, additionally suggest the neurotoxicity of these compounds [17].

As mentioned, some previous studies suggest a link between phthalates' exposure to ADHD [18], ASD [19], and other cognitive or behavioral impairments [20]. Due to the known circumstances, studies of neurobehavioral outcomes in humans following phthalate exposure are limited and need following biochemical investigation. Some authors reported that prenatal exposure to phthalates has detrimental consequences on both functional and structural plasticity aspects of the hippocampus. The precise molecular and physiological mechanisms of phthalates' CNS toxicity are still unknown and need intensive further biochemical study in the context of the link with increased prevalence of hyperactivity, autism spectrum disorders, and other behavioral disturbances in children [21]. It was shown that prenatal exposure to some phthalates (DEHP, DBPs, DEP, and BBzP) affects cognitive and psychomotor development and induces significant changes in internalizing and externalizing behaviors, attention process, recognition memory, social responsiveness, and visual-spatial abilities of children, often clinically diagnosed as developmental disabilities including autism and ADHD [16].

Among the most often used phthalates found in a wide range of industrial and consumer plastic products, DEHP is in the top five. The list of products, among others, also includes medical tubing and medical bags. For the last decades, the high potential of DEHP-induced reproductive and developmental toxicity has been approved [22]. However, only a few studies have identified the possible mechanism of DEHP neurotoxicity.

Some researchers suggest that the target structure for DEHP neurotoxicity is the hippocampus [23], but studies on other brain structures were not conducted. Researchers discovered that continuous exposure of adult male mice to ambient concentrations of DEHP alone or with a phthalate mix led to impaired short-term memory in spatial, temporal order, and novelty tasks. The apoptosis of hippocampal neuronal cells accompanied these cognitive deficits. The behavioral changes have been associated with the decreased density of the dendritic spine and protein levels of postsynaptic markers in the hippocampus [24, 25]. Recently, it was shown that DEHP prenatal exposure induces activation of the PI3K/Akt/mTOR signaling pathway in the offspring's testis [26]. Since the same signaling pathway was recently reported as a key player in the development of ASD and is also implicated in the pathogenesis of Alzheimer's disease, Parkinson's disease, epilepsy, depression, schizophrenia, and bipolar disorder [27], we have proposed it as one of the candidates for DEHP neurotoxicity, too. Signaling duration of PIP3 – the main PI3K/Akt/mTOR cascade trigger is regulated by phosphatase and tensin homolog (PTEN), which counteracts PI3K activity. Studies report PTEN as one of the main genetic factors that are involved in the pathophysiology of autism spectrum disorders (ASD) [28]. Specific modes of PTEN's subcellular localization in different compartments provide its involvement in mediating the profusion of cellular responses. It was shown that the mode of membrane PTEN recruitment and downstream activity is regulated by the lipid composition of membranes in several neural cells, including cortical neurons and oligodendrocytes [29–31].

Based on the observations, we suggest that PTEN localization and activity in the target brain region should mediate DEHP prenatal neurotoxicity. Early stages of neurodevelopment are especially vulnerable, and prenatal exposure can induce irreversible changes linked to further behavior and cognitive disorders. Recent studies have demonstrated significant sex differences in the regulation mechanisms of the hippocampal Akt pathway, suggesting that the main processes in the hippocampus are less dependent on endocrine status in males compared to females [32]. The main goal of our research was to assess the effect of DEHP prenatal subchronic exposure on the behavior and learning ability of male rats, identify the primary target brain structure/s of neurotoxic action, and determine the involvement of the PTEN signaling pathway and its intracellular localization in the molecular mechanism of phthalates' neurotoxicity of the immature male brain.

2. Materials and Methods

2.1. Animals

2.1.1. Prenatal exposure

Wistar rats were sourced from the breeding colony at the I. Beritashvili Center of Experimental Biomedicine (Tbilisi, Georgia). Male and female Wistar rats weighing 350-400 g and 200-250 g were selected for mating. A forced swimming test was conducted to rule out endogenous depression. Animals were housed in groups under controlled temperature conditions (22 ± 2°C) with a 12-hour light/dark cycle and had ad libitum access to food and water. Selected females received an oral dose of di(2-ethylhexyl) phthalate (DEHP) at 500 mg/kg/day, dissolved in water, from the fetal to the neonatal period. The dosage was determined based on previous studies [33–35]. The use of 500 mg/kg oral DEHP in drinking water for prenatal rat studies reflects its purpose to evaluate high-dose

exposure effects relevant to human health. Rats metabolize DEHP more rapidly than humans, requiring higher doses to achieve comparable internal exposures. This dose enables the identification of developmental and reproductive toxicities during critical prenatal windows, mimicking worst-case human exposure scenarios (e.g., medical procedures or industrial settings).

To evaluate the impact of gastrointestinal exposure to DEHP, we administered the compound via drinking water to ensure a consistent daily dosage. DEHP exposure commenced three days post-pairing, shortly after mating confirmation. Females in the control group received plain water. Pregnancy in all females was monitored by daily tracking of females' body weight. The average gestational period was 20-25 days from the first mating, with no differences observed between groups. Animals were housed in polycarbonate cages with stainless-steel lids, wood shavings for bedding, and glass water bottles to minimize external phthalate contamination. Post-delivery, exposure of offspring (10 ± 2 pups per dam) was minimized by discontinuing DEHP administration to mothers during lactation.

After 3 weeks, pups were separated from their mothers and placed in the conditions described above until they became ten weeks old. Offspring were housed in cages (5 rats per cage), provided with food and water available ad libitum, and maintained under conditions of 20-22˚C and appropriate humidity on a 12-h light/dark cycle. No differences in pups' weight after delivery between groups were observed, but weight gain at 10 weeks old age was 10±3% more in DEHP prenatally treated rats. Male rats were selected from the offspring litter (N=16 for the control group and N=24 for the DEHP group). The subsequent experiments were conducted exclusively on these male offspring and did not include any data on the parents or female offspring. Female pups were excluded from the described experiments and designated for further endocrine status analysis.

2.1.2. Ethics approval of research

Animal care during experimental procedures was carried out under the recommendations of the Ilia State University Research Projects Ethics Commission (decision letter R/294-23) and following the Council of Europe Directive 2010/63 / EU for animal experiments.

2.2. Behavioral tests

After the offspring of the initial test male rats reached 10 weeks of age, the following behavioral tests were performed:

Open field test: an experimental test used for the general cognition and anxiety level assessment. The test was performed in a 1 m^2 white square. On the bottom, we've drawn 25 small cubes with a marker. Animals were placed in the area and were given 10 minutes in a quiet room and freedom of movement while being recorded. Recordings were analyzed for the following parameters: rearing, grooming, line crossing, and time spent in the center [36].

Novel object recognition is a widely used behavior assessment for different aspects of learning and memory. Assessment takes place in 50 cm^2 squares and takes three days to finish. On the first day of the test, objects go through a habituation period for 5 minutes. This is essential for familiarization with the area. On the second day, two identical objects were placed on the diagonal opposite each other. On the third day, one of the objects was changed and had a different color and shape but a similar size. Second and third-day experiments were recorded. Recordings were analyzed mechanically for the following parameters: latency to the objects, preference for the new object, exploration of the objects, and transition between objects [37].

Elevated Plus Maze: used for evaluation of neurobiological anxiety. The apparatus comprises a plus-formed maze, which is elevated above the floor. It has two oppositely positioned closed black-colored arms, two open white-colored arms, and a center area. During this test, the following parameters were analyzed and assessed: time spent in the open space, time spent in the closed space, and time spent in the center area [38].

2.3. Decapitation and brain structures' isolation

After the performance of behavioral tests, the rats were euthanized by rapid decapitation using a hand-operated guillotine. Decapitation is chosen for euthanasia to keep brain architecture for unique regions and avoid chemical contamination for further analysis according to AVMA Guidelines for the Euthanasia of Animals, 2020 Edition [39]. After each use, the decapitation equipment was cleaned of any biological fluids with ethanol and water and then wiped. Rat brains were isolated on ice, and the brain structures of the hippocampus, prefrontal cortex, and hypothalamus were extracted. Those structures have been chosen based on previously performed studies on phthalates [40].

2.4. Subcellular fractioning

The presented experiments used the brain structures of four control male rats and four DEHP-exposed male rats. Subcellular fractions were obtained according to Won et al. [41]. Freshly isolated brain structures were immediately homogenized in a Dounce homogenizer with ice-cold TEVP buffer (10mM Tris-HCl (pH 7.5), 5mM EDTA, 1mM DTT, 1mM PMSF, protease inhibitor cocktail), containing 320 mM Sucrose. This and all the following stages were performed at 4^0C temperature. Homogenate was centrifuged at 1000 x g for 10 min, and the obtained supernatant was centrifuged again at 12000g for 20 min. The pellet was resuspended with twice the volume of TEVP buffer containing 35.6 mM Sucrose, incubated for 30 min, and centrifuged at 25 000 x g for 20 min. To extract the extra-synaptic (non-PSD enriched) protein fraction, the pellet was resuspended in a 3-fold volume of ice-cold TEVP buffer containing 1 % Triton X-100. Solubilization was performed at 4 °C for 15 min, followed by centrifugation at 20,000 × *g* for 30 min. The experiments used the supernatant as an "extra-synaptic protein fraction." For solubilization of the synaptic (PSD-enriched) protein fraction, the pellet was resuspended in a 3-fold volume of TEVP buffer containing 1% SDS, followed by incubation for 2 h at 4 °C with continuous gentle vortexing. Finally, the samples were centrifuged at 100,000 × *g* for 30 min, and the supernatant was stored and used as a "synaptic protein fraction." All solubilized proteins were stored at -80 ^{0}C until further experiments.

2.5. Western blotting

Western Blotting analysis was performed using the Abcam® Western Blot protocol [42]. Aliquots of the cytosol-

ic, mitochondrial, and plasma membrane protein fractions (containing ≈ 60 µg, 30 µg, and 40 µg of total protein, respectively) were dissolved in equal volumes and loaded on SDS gels (4-12%) and electrophoresed. The separated proteins were then transferred onto 0.45 µm nitrocellulose membranes using electroblotting. Membranes were stained with Ponceau S solution to confirm correct sample loading and efficient protein transfer. Subsequently, the membranes were blocked with 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) and incubated for 1 h with the following antibodies against the corresponding antigens:

In the mitochondrial fractions - (Mitofusin-2 [ab124773]; Drp-1 [sc-101270] and Nrf1 [sc-721])

In the cytosol of the hippocampus and prefrontal cortex structures (p-Akt [sc-377556], mTOR [sc-517464], p-mTOR [sc-293133] and PTEN [sc-7974]).

In the synaptic fraction: (IP3R [sc-377518], (PTEN [sc-7974], NMDA ƛ1 [sc-518053], NMDA ε1 [sc-1468] and NMDA ε2 [sc-1469]).

All primary antibodies were purchased from Santa Cruz Biotechnology Inc. (USA). After incubation, the membranes were washed in TBST and probed with species-appropriate peroxidase-conjugated secondary antibodies at 20^0C for 1 h. After further washing in TBST, these antibodies were detected by enhanced chemiluminescence autoradiography (ECL kit, sc-2048; Santa-Cruz Biotechnology). The blots were exposed to autoradiograph films (Amersham). Obtained films were then digitalized by photo equipment, and their intensities were quantified using Image Lite Studio software version 5.2.5 (Li-Cor). β-actin antibody [sc-32273] was used as a loading control in corresponding fractions.

2.6. The Phosphorylation pathway profiling array

The phosphorylation pathway profiling array was performed using a Multi-Pathway Profiling Array C55 Kit (Raybiotech, cat# AAH-PPP-1- 4 – Akt pathway), which followed the manufacturer's protocols. First, membranes were incubated with a blocking buffer at 25 °C for 30 minutes, followed by an overnight incubation at 4 °C with 1 mL of cytosolic fractions of hippocampus cells (control and DEHP groups). The membranes were then incubated with Detection Antibody Cocktail at room temperature for 2 hours on a shaker, followed by a 2-hour incubation with secondary horseradish peroxidase (HRP)- labeled anti-rabbit antibody also at room temperature. ECL kit sc-2048 from Santa Cruz Biotechnology was used to detect signals through enhanced chemiluminescence autoradiography. Autoradiograph films (Amersham) were used to expose the blots. The intensities of the acquired films were then measured using Image Lite Studio software, version 5.2.5 (Li-Cor) after they had been digitalized by photo equipment. Two biological replicates were performed, and the average expression levels were compared between the treatment and control samples. Results were normalized using positive control and background

2.7. Total protein amounts

Total protein amounts of the obtained fractions were determined using a BCA protein assay kit (sc-202389; Santa Cruz Biotechnology) according to the manufacturer's protocol.

2.8. BDNF quantitative analysis

BDNF quantitative analysis in the cytosol fraction was performed using the relevant kit (BDNF Human ELISA Kit [ab99978], Biotech) following the manufacturer-provided protocol.

2.9. Caspase-3 activity assay

Caspase-3 activity assay was performed in the cytosol fraction using the relevant kit (Caspase-3 Assay Kit [ab39401], Biotech).

2.10. Statistical analysis

A one-way analysis of variance (ANOVA) was conducted to analyze the data collected from behavioral tests. The analysis was performed using ANY-maze Video Tracking Software 60000. Tukey's post hoc multiple comparison test was applied to identify statistically significant differences between groups.

The optical density of bands and spots was analyzed using LI-COR Image Studio Software. The software facilitated accurate quantification by measuring the intensity of signals in the regions of interest. Background subtraction was applied to ensure the measured values represented true signal intensity. Data were normalized to loading controls to account for variability between samples. Groups were compared by planned comparisons, using two-tailed t-tests and one-way ANOVA.

3. Results

3.1. Behavior

Analysis of parameters of the Open Field test revealed that DEHP prenatal exposure in male rats significantly increases anxiety and depression levels compared to the relevant controls. Rearing quantity in the phthalate group was increased by 78%; line crossing was not increased significantly, whereas the grooming quantity was decreased by 29% relative to the control, which may indicate a higher depression background (Figure 1). The time in the center for the DEHP group was also decreased, which also suggests increased anxiety and fear levels.

In the case of Novel Object Recognition, the preference of the 2nd object in DEHP pretreated rats was 48% less than in the control group, which indicates lower recogni-

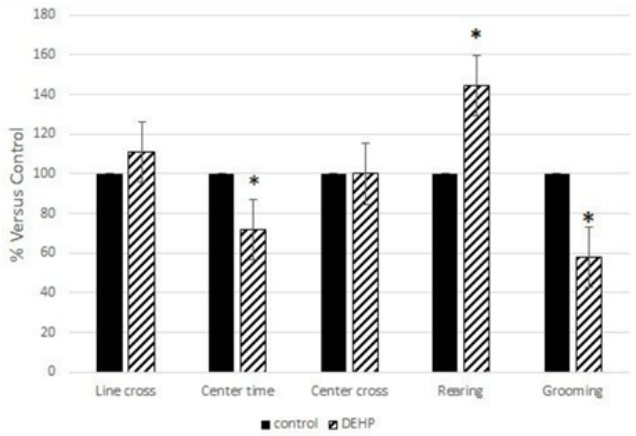

Fig. 1. The mean values of open field crossings for control and DEHP prenatal exposed groups of male rats (N=16 for control, and N=24 for DEHP group). The data are presented as % versus control group ± standard error (SE). One-way ANOVA test for different treatments; overall, the test was significant at P<0.05.

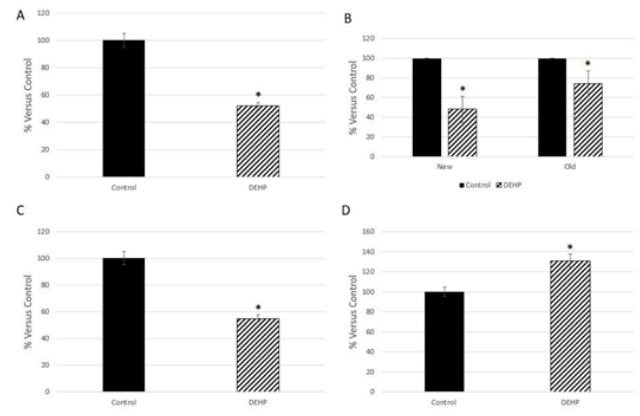

Fig. 2. Effect of DEHP prenatal exposure on the Novel Object Recognition test. (N=16 for control, and N=24 for the DEHP group). (2A) Preference of the new object on the 2nd day; (2B) Exploration time of both objects on the 2nd day; (2C) Latency to any object on the 2nd day; (2D) Transition count between the objects on the 2nd day. Values are mean ± standard error (SE). One-way ANOVA test for different treatments; overall, the test was significant at P<0.05.

tion memory and loss of interest (Figure 2a). The exploration time of the new object on the 2nd day was 52%, and in the case of the familiar object, it was 26% lower in the DEHP group relative to the control group (Figure 2b). Latency in DEHP pretreated objects was decreased by 45% versus control (Figure 2c). We've also assessed the transition count from one object to another in both groups, which revealed that the phthalates group rats transitioned 31% more times than the control group (Figure 2d). This data shows increased anxiety levels and aimless, hasty movement.

The Elevated Plus Maze test revealed that both groups spent less time and rarely visited open or middle spaces than closed ones. The time spent in the open space for the phthalate and control groups was almost the same; the average number shows only a 2-second difference. Statistically approved changes were found only in the number of visits to the open space - it was increased by 21% in the DEHP pretreated group compared to the control (Figure 3). The data received in this test once again proves elevated anxiety levels after receiving phthalates [38]

3.2. Signaling pathways

Based on the obtained results and previous data on neurotoxicity target structure [43] three brain regions (hippocampus, prefrontal cortex, and hypothalamus) were isolated and homogenized, and subcellular fractions were obtained according to the abovementioned protocols.

In the first series of experiments, to assess the apoptosis and neurotoxicity induced by DEHP prenatal exposure, we have determined the caspase-3 activity and BDNF content in the cytosol fraction of brain regions. Obtained data showed that caspase-3 activity in DEHP-pretreated rats was increased in the hippocampus and prefrontal cortex by 51% and 77%, respectively, compared to the control samples (Figure 4A). BDNF content analysis revealed significant changes in the hippocampal cytosol fraction compared to the control group samples (Figure 4B).

Based on the obtained data, two brain structures – the hippocampus and prefrontal cortex were selected for the following experiments to assess DEHP-induced changes in Akt/mTOR pathway and PTEN signaling. Analysis of data obtained by Western blotting experiments revealed that prenatal exposure of male rats to DEHP enhances Akt/mTOR pathway activity due to an increase in the levels of mTOR and its phosphorylated form (Figure 5A) in the

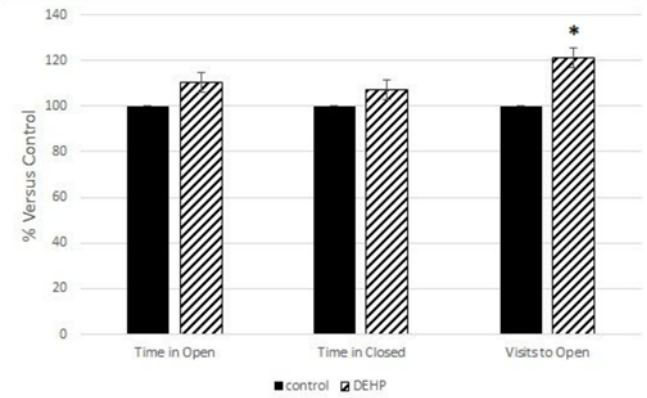

Fig. 3. Effect of DEHP prenatal exposure on Elevated Plus Maze test in male rats. (Mean of the N=16 for control, and mean of the N=24 for the DEHP group)Values are expressed as % versus control group ± standard error (SE). A one-way ANOVA test was used for analysis; overall, the test was significant at P<0.05.

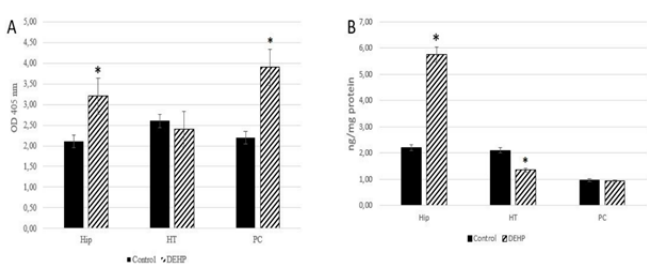

Fig. 4. Effect of DEHP prenatal exposure on (A) caspase-3 activity and (B) BDNF content in the selected brain structures (Hip – Hippocampus, HT – Hypothalamus, PC – Prefrontal Cortex). Results are expressed as mean ± SEM (n = 4). Statistical analysis was carried out by one-way ANOVA *p < 0.05.

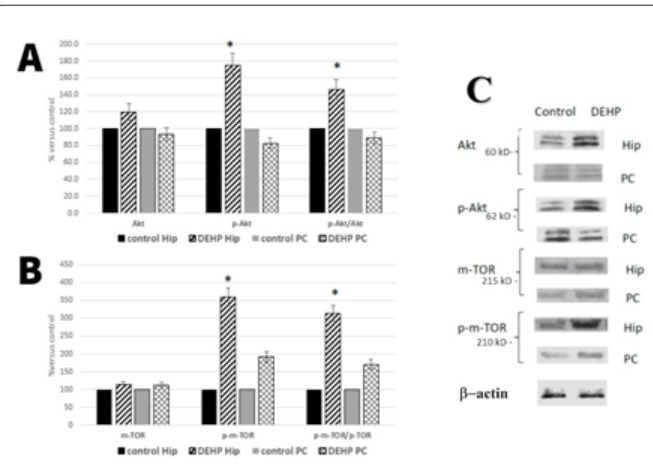

Fig. 5. The protein level of non-phosphorylated, phosphorylated forms and their arbitrary ratio of mTOR (A) and Akt (B) in the cytosol fractions of the hippocampus and prefrontal cortex of control and DEHP exposed rats, as measured by western blotting. The bands (one representative of each is displayed in C) were acquired, and their intensities were quantified using Image Lite Studio software. Data are normalized and expressed as mean optical density ± standard error of the mean (SEM). (p < 0.05).

cytosolic fraction of both structures and increase of Akt phosphorylation only in hippocampal cytoplasm (Figure 5 B).

These changes are accompanied by a significant decrease in the amount of cytosol PTEN, especially in the hippocampal fractions (Figure 6).

All the above-mentioned changes were more conspicuously expressed in hippocampal fractions, which approved the suggestion about primarily targeting this brain structure for phthalates' action.

To approve the changes in the PTEN-Akt associated pathway and explore phosphorylation changes of other proteins in hippocampi caused by DEHP prenatal treatment, the cytosolic fractions from control and DEHP-treated brains' hippocampi were subjected to phosphorylation pathway profiling array. As shown in Figure 7, phosphorylated PTEN and PDK1 (Phosphoinositide-dependent kinase 1) have the most significantly reduced signal. In contrast, the content of phosphorylated Akt, AMPKa (5′-AMP-activated protein kinase catalytic subunit alpha), and pro-apoptotic protein BAD (BCL2 Associated Agonist Of Cell Death) was increased in DEHP-treated hippocampi compared to respective control.

A decrease in cytosol PTEN in neural cells could result from translocation and association with the plasma membrane. Therefore, in the next series of experiments we have examined the content of PTEN in the solubilized fraction of the hippocampal synaptic membranes. The obtained data shows that DEHP prenatal exposure causes a significant increase in PTEN content in the synaptic membrane compared to the respective control (Figure 8). It was shown previously that membrane-localized PTEN physically interacts with GLUN1/GLUN2B complexes of NMDA receptors within hippocampal neurons and depresses synaptic transmission through NMDA receptor (NMDAR)-dependent long-term depression (LTD) [44]. Western blotting experiments of the hippocampal synaptic fractions of both animal groups showed a significant increase in the NMDA receptors GluN1 subunit in the DEHP-pretreated group (Figure 8). Also, the protein level of GluN2B was raised in this group compared to the control group samples (Figure 8). No significant changes were found in the GluN2A subunit content in synaptic membranes (Figure 8).

Some recent findings suggest PTEN's association with the mitochondria's outer membranes in response to some apoptotic stimuli [45] and its potential role in regulating mitophagy and mitochondrial dynamics [46, 47]. The link between the deletion of PTEN and increased MFN2 expression that results in rescued mitophagy flux via the AMP-activated protein kinase (AMPK)–cAMP response element-binding protein (CREB) pathways was shown recently [48]. To assess the involvement of PTEN-induced changes in mitochondrial dynamics in DEHP neurotoxicity, the content of three key molecules – DRP1, MFN2, and Nrf1 was estimated. We have found that prenatal exposure to DEHP induces a decrease of MFN2 in the hippocampus compared to the respective control samples (Figure 9). No statistically significant changes in the case of DRP1 and Nrf1 were revealed.

Therefore, experiments showed that prenatal oral exposure to DEHP induces changes in cognitive behavior and space memory that are accompanied by elevation of BDNF, enhancement of Akt/mTOR pathway in the cytosol, PTEN/NMDA GluN2B signaling in synaptic membranes, changes of MFN2-mediated mitochondrial fusion in the hippocampus of male offspring.

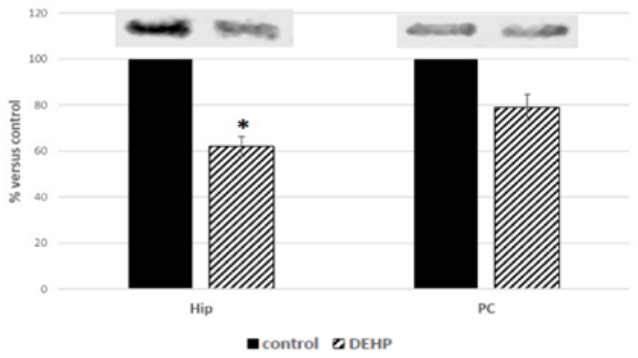

Fig. 6. As measured by western blotting, the protein level of PTEN in the cytosol fractions of the hippocampus (Hip) and prefrontal cortex (PC) of control and DEHP-exposed male rats. The bands (one is displayed over the respective chart) were acquired, and their intensities were quantified using Image Lite Studio software. Data are normalized and expressed as mean optical density ± standard error of the mean (SEM). ($p < 0.05$).

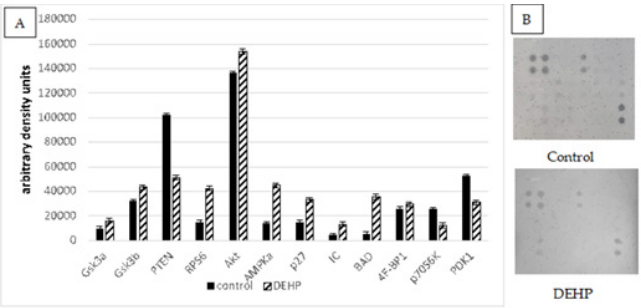

Fig. 7. (A) The graph represents modulated phosphorylated protein expression in arbitrary density units obtained by chemiluminescence and autoradiograph detection. Data are presented as the mean ± standard deviation of three independent experiments. One way–ANOVA= $p < 0.05$; (B) Images of nitrocellulose membrane showing differential protein expression of the cytosolic fraction of control and DEHP-treated hippocampi.

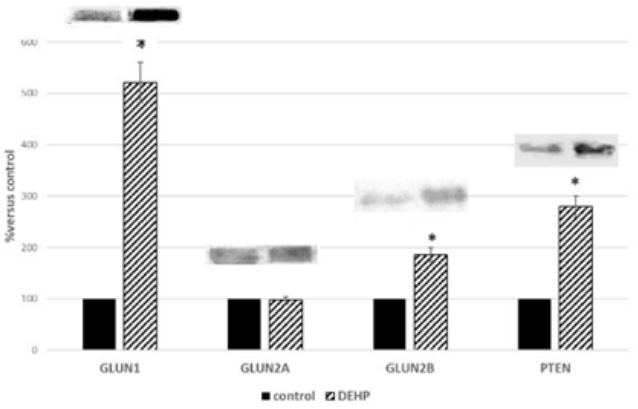

Fig. 8. Protein levels of GluN1, GluN2B, GluN2A, and PTEN in fractions solubilized from the synaptic plasma membrane of the hippocampus of control and DEHP-exposed rats, as measured by western blotting. The bands (one is displayed over the respective chart) were acquired, and their intensities were quantified using Image Lite Studio software. Data are normalized and expressed as mean optical density ± standard error of the mean (SEM). ($p < 0.05$).

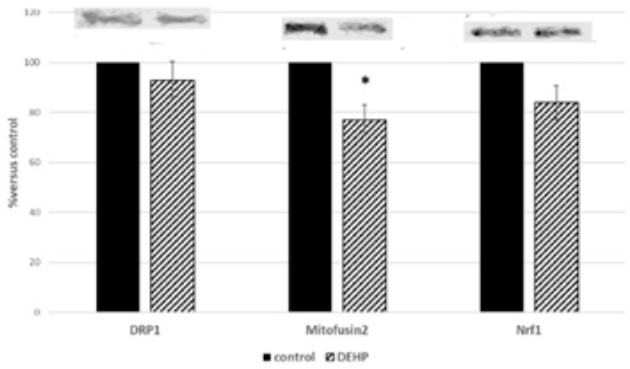

Fig. 9. The protein level of DRP1, MFN2, and Nrf1 in the hippocampal fractions of control and DEHP-exposed rats, as measured by western blotting. The bands (one is displayed over the respective chart) were acquired, and their intensities were quantified using Image Lite Studio software. Data are normalized and expressed as mean optical density ± standard error of the mean (SEM). ($p < 0.05$).

4. Discussion

Since the hippocampus is responsible for a significant portion of altered functions (neural plasticity, cognitive flexibility, anxiety-like behavior, learning, and memory), its vulnerability to phthalates was suggested. Few recent studies showed the link between prenatal exposure to DEHP in male mice and neuronal loss in the hippocampus, accompanied by oxidative stress and neuroinflammation. These changes are strongly correlated with elevated anxiety behavior and impaired recognition memory [40]. The impaired dendritic complexity in the hippocampus of perinatally exposed to DEHP male rats, particularly in CA1 pyramidal neurons, was also described [49].

The analysis of behavioral and biochemical changes induced by prenatal DEHP exposure initially focused on male offspring. This selection was primarily based on the fact that male rats exhibit more excellent stability in their response to toxins and are less influenced by hormonal fluctuations than females. Additionally, male rats display a relatively consistent pattern of physiological and social behaviors, reducing confounding variables and thereby enhancing the reliability and comparability of the study results. [50, 51].

Our experiments revealed that prenatal oral exposure to DEHP at a concentration of 500 mg/kg/day induces significant changes in neurodevelopment of male-offspring that can be expressed even 10 weeks after birth when exposure to phthalates is stopped. Increased anxiety is expressed in enhanced rearing, line crossing, hasty movements, and decreased grooming quantity. These changes are accompanied by lower recognition memory and loss of interest in new object recognition. Since increased anxiety is often associated with the pathological activity of the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamus, together with the hippocampus and prefrontal cortex, were selected for further assessment of the damage. The caspase-3 assay showed that the hippocampus is the main target for DEHP neurotoxicity. Elevated BDNF content in the hippocampus also indicated the principal role of this structure in behavior changes. The change in BDNF level is often connected with neurotoxicity and neurodegenerative diseases [52, 53]. Cominski et al. [54] show that the hippocampus implicates anxiety disorders and learning avoidance, which additionally supports our suggestion. Additionally, a decreased number of hippocampal pyramidal neurons in DEHP prenatally exposed objects was observed [40].

The exact mechanisms that explain the adverse effects of DEHP on the neurodevelopment of early life stages are unclear. Since DEHP converts to its active monoester metabolite mono (2-ethylhexyl) phthalate (MEHP) and penetration of both phthalates into the placenta is high, MEHP could mediate the neurotoxic effect, also [55]. The recent findings revealed that DEHP exposure induced enhanced proliferation and metastasis of neuroblastoma cells and liver cancer cells, which involve activation of PI3K/Akt/mTOR signaling [56]. Increased PI3K/Akt/mTOR signaling has also been found in the F1 and F2 generations of maternal exposure to DEHP [57]. Our experiments also support the proposed mechanism of toxicity and show selective enhancement of the Akt/mTOR pathway in the hippocampus. Since the activity of Akt/mTOR dramatically depends on the PI(3,4,5)P3 concentration, PTEN, which dephosphorylates PI(3,4,5)P3, antagonizes the PI3-Kinase signaling pathway and serves as a negative regulator of Akt in the rat hippocampus [58].

PTEN is a lipid and protein phosphatase involved in the regulation mechanisms of diverse cellular processes. The transient association of cytosolic PTEN with the plasma and other membranes changes its downstream effectors and cell response. The decreased level of PTEN found in DEHP-exposed rats in the hippocampus is possibly a key mechanism underlying impaired cognitive behavior and anxiety. The gene encoding phosphatase and TENsin homolog deleted on chromosome TEN (*PTEN*) is a well-recognized syndromic risk allele for autism spectrum disorder (ASD), a neurodevelopmental disorder defined by deficits in two core symptom domains: social communication/interaction and restricted/repetitive behavior [59–62]. Since PTEN participates in establishing a PIP3/PIP2 gradient, the proper localization of this molecule at the membrane can be considered a critical factor for the recruitment of essential components necessary for the formation of growth cones and dendritic spines. This opinion is additionally supported by the evidence that PTEN is present in most neurites, axons, and dendrites. In neurons, PTEN may be sequestered away from the cell membrane and selectively recruited to the membrane under certain conditions.

Diverse mechanisms, including posttranslational modifications, protein-protein interactions, and lipid rafts of membranes, regulate the subcellular localization of PTEN. Thus, PTEN function depends not only on the enzymatic activity but also on the spatial distribution of this molecule. For example, the study using AD models has shown that Aβ provokes this shift toward synaptic depression by triggering the access to and accumulation of PTEN in the postsynaptic terminal of hippocampal neurons [63]. Analysis of PTEN content in hippocampal synaptic membranes of control and DEHP-exposed rats showed increased synaptic PTEN protein levels compared to the respective controls. Therefore, prenatal exposure to DEHP provides relocation of PTEN from the cytosol to synaptic membranes, where PTEN interacts with NMDA glutamate receptors and participates in long-term depression (LTD) [44, 63, 64].

PTEN accumulation in the postsynaptic compartment was shown in response to Aβ [63]. PTEN may physically

interact with the GLUN1/GLUN2B complex of NMDA receptors within hippocampal neurons, resulting in synaptic depression [44, 63, 65]. Our further investigation of the protein content of the hippocampal synaptic membrane has also shown an increase in GluN2B-containing NMDA receptors induced by prenatal exposure to DEHP.

It was shown that during some pathologies and in response to apoptotic stimuli, PTEN's association with mitochondria membranes has also been demonstrated in hippocampal neurons. [45]. A possible mechanism of mitophagy's regulation by PTEN should involve dephosphorylation of pSer72-RAB7A [46, 47] and a decrease of MFN2 by the AMPK-CREB pathway [48]During the experiments, we studied the DEHP-induced changes of key players of mitochondrial dynamics—DRP1 (a marker of fission), MFN2 (a marker of fusion), and Nrf1 (involved in mitochondrial biogenesis). We showed decreased levels of MFN2 that could be provoked by PTEN relocation to mitochondria. Mfn2, a key marker of mitochondrial fusion, is linked to ER-stress tolerance in neurons and astrocytes. Its downregulation has been observed in conditions such as excitotoxicity and delayed neuronal death. Since mitochondrial fusion is regarded as a potential compensatory mechanism that helps protect cells from apoptosis, reduced Mfn2 levels may impair the cell's ability to mitigate stress and maintain survival [66, 67].

The possible mechanism underlying the DEHP effect on PTEN localization in neural cells could possess changes in membrane lipids [68, 69]. Due to its high lipophilicity, exposure to chemicals such as DEHP may alter the fatty acid composition in the immature brain and consequently change lipid signal transduction pathways, resulting in neurodevelopment impairment.

Considering all the obtained data, we can assume that prenatal oral exposure to DEHP in a selected concentration induces irreversible changes in brain structures of the male offspring, primarily in the hippocampus, that underlie significant alterations in cognitive behavior and enhanced anxiety. The molecular mechanism of DEHP-induced neurotoxicity in the maturing brain involves changes in PTEN subcellular location, which suppresses Akt/mTOR signaling, enhances GluN2B NMDA-mediated synapse depression, and decreases mitochondrial fusion.

Abbreviation

Akt – Protein Kinase B; AMPK – AMP-activated protein kinase; ASD – Autism Spectrum Disorders; BAD - BCL2 Associated Agonist Of Cell Death; BDNF – Brain-Derived Neurotrophic Factor; CREB – cAMP Response Element-Binding Protein; DEHP – Di(2-ethylhexyl) phthalate; Drp1 – Dynamin related protein 1; GluN1 – NMDA Receptor Subunit 1; GluN2A - Glutamate [NMDA] receptor subunit epsilon-1; GluN2B - Glutamate [NMDA] receptor subunit epsilon-2; LTD – Long-Term Depression; MEHP – Mono(2-ethylhexyl) phthalate; MFN2 – Mitofusin 2; mTOR - The mammalian target of rapamycin; NMDA – N-Methyl-D-Aspartate; Nrf1 – Nuclear respiratory factor 1; PDK1 - Phosphoinositide-dependent kinase 1; PIP3 - Phosphatidylinositol (3,4,5)-trisphosphate; PTEN – Phosphatase and Tensin Homolog

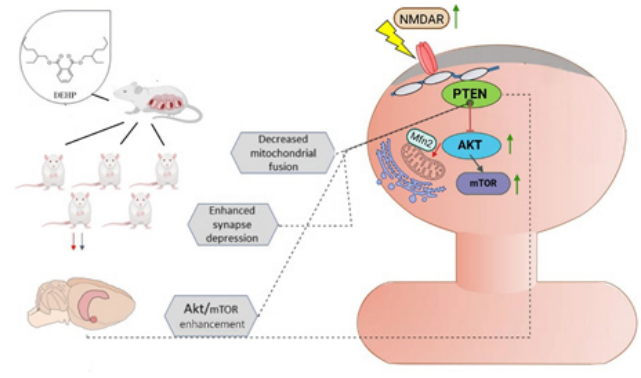

Fig. 9. Proposed mechanism of DEHP prenatal exposure-induced hippocampal neurotoxicity.

Author contributions

Natalia Kiknadze: Carried our main experiments with animal behavior, carried out animal euthanasia, decapitation, and brain homogenization, made data analysis, and participated in paper writing. Elene Zhuravliova: Proposed the central hypothesis, designed the main stages of the research plan, organized the study, made biochemical analysis, and prepared the visual part of the paper. David Mikeladze *: Made interpretation of obtained data, prepared introduction and discussion part of the paper.

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Institutional review board statement

Animal care during experimental procedures was carried out following the recommendations of the Ilia State University Research Projects Ethics Commission and with the Council of Europe Directive 2010/63 / EU on animal experiments.

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Conflicts of interest

The authors declare no conflict of interest.

Consent for publication

All authors have read and approved the final manuscript for publication.

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